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Shedding the load: moulting as a cause of variability in whole-body metal concentrations

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ABSTRACT

Moulting is a biological process shared by aquatic macroinvertebrates, but while the exoskeleton is believed to be a major sink of metal pollutants, the contribution of the moulting of the crustacean exoskeleton to total accumulated metal concentrations is insufficiently considered. We present a conceptual, qualitative model that illustrates the impact of moulting on the whole-body burden of an unspecified metal analyte in a hypothetical moulting invertebrate. The model

demonstrates that moult stage is a contributor to the whole-body pollutant concentration, and that this introduces a temporal component even in steady-state exposure conditions. The applicability of this model is illustrated by comparison to published results of pre- and post-moult accumulations. A solution for reducing this variability in the measurement of whole-body metal concentrations is presented, and its potential application to both *ex-situ* and *in-situ* studies of biomonitor species is discussed.

Key Words: aquatic environment, bioaccumulation, body burden, crustaceans, ecdysis, measurement errors, exoskeleton, macroinvertebrates, metal pollution

INTRODUCTION

Biomonitoring is defined as the use of an organism, whether whole, in part, or communal, to determine the quality of the environment, and it is commonly used as a method of detecting and quantifying contaminants present in the environment (Markert, 2007). Biomonitoring is generally employed for the quantification of environmental pollutants, wherein a measurable parameter may be the abundance of an identified biomonitor species in a geographical area, or under a defined set of conditions (Ketelaars & Frantzen, 1995; Bonada *et al.,* 2006;).

A primary application of an aquatic biomonitor is the measurement of bioavailability and uptake of trace metals in the environment, and hence an indication of environmental concentrations (Johnson *et al.,* 1993; Flessas *et al.,* 2000). Trace metals may enter the aquatic environment by means of natural processes, such as metals leaching into rivers following a forest fire in an adjacent location, or through anthropogenic disturbance (Richardson *et al.,* 2001). In many instances, there is a higher ratio of metals entering the hydrosphere as a result of

anthropogenic activities than of metals from natural sources entering waterways (Callender, 2003).

A distinction must be made between total and bioavailable pollutant concentrations in order to appreciate the advantage of biomonitoring over direct measurement of the pollutants. Various physico-chemical processes can render a pollutant biologically inert. These can be divided into environmental processes, that influence the "environmental availability," and internal biological processes within an organism, that influence the "toxicological bioavailability" of the pollutant (Peijnenburg *et al.*, 1997). While total pollutant concentrations can be determined directly through chemical analysis, accounting for the concept of bioavailability requires either the application of measured environmental parameters to a model of the environmental processes (the *a priori* approach, often the application of partition coefficients to measured total pollutant concentrations), or direct measurement of accumulated concentrations within the organism of interest (the *a posteriori* approach). The classification of pollutants according to their bioavailability and toxicity, such as the Priority Substances List of the Water Framework Directive and subsequent amendments (European Commission, 2000, 2008), is a similar approach to the former. Biomonitors exemplify the latter, as the measured biological concentrations are the result of both the environmental availability and toxicological bioavailability processes, and, therefore, offer an insight into the results of these processes without requiring knowledge of the processes themselves. This approach avoids the need to simplify such processes, so long as the biomonitor is chosen such that the toxicological bioavailability encountered is representative for the ecosystem under study.

The biomonitoring of bioavailable pollutant concentrations using biomonitor organisms also presents another advantage. Metal pollutants are often concentrated within the organism of

interest, which can allow for the observation of concentrations of contaminants that would otherwise be difficult to detect at background concentrations, especially at a high spatial or temporal frequency that would otherwise be difficult and prohibitively expensive to carry out using analytical methods (Bryan & Darracott, 1979). This concentration behaviour relaxes the limits of detection and allows the quantification in the organism what would rarely be detected in the water (Phillips, 1977).

There is still some debate around the extent to which crustaceans uptake and bioaccumulate trace metals, and reproducibility of measured metal concentrations appears to be the exception, rather than the norm (Depledge & Rainbow, 1990; O'Callaghan *et al.,* 2019). This is compounded by the uncertainty regarding the most likely sources, uptake pathways or sites of bioaccumulation within the organism of interest (Fleming & Richards, 1982; Elangovan *et al.,* 1999; Van Hattum *et al.,* 1999; Robinson *et al.,* 2003; Santoro *et al.,* 2009). Several models have been proposed for the processes of uptake, bioaccumulation, and excretion of various pollutants in freshwater macroinvertebrates (Rainbow & Luoma, 2011; Awrahman *et al.,* 2015), a task made more complicated by the varying behaviour of different metal analytes and potential interactions between said metals (O'Callaghan *et al.,* 2019). A survey of these models, however, indicates that periodic moulting of the exoskeleton, a process common to many invertebrate species (Lebrun *et al.,* 2011), may not have been adequately accounted for in regard to trace metals loss from the whole organism upon renewal of the exoskeleton.

Failing to take into account exoskeletons could therefore be a substantial source of variability in measurement of the bioavailability of trace metals or in biomonitoring programs. Previous studies have shown that the moulted exoskeleton may contain sizable concentrations of bioaccumulated pollutants, pointing to ecdysis as being a possible pathway through which

significant portions of the accumulated substance may be shed or lost from the organism (Miramand *et al.,* 1981; Hall, 1982; Topcuoğlu *et al.,* 1987; Rauch & Morrison, 1999). Moulting may ultimately influence survival of an organism, as periodic moulting may reduce or maintain pollutant concentrations below critical concentrations for the organism (Bryan & Darracott, 1979; Bergey & Weis, 2007). Furthermore, in the context of quantifying the accumulated concentrations, accuracy and applicability of existing pollutant uptake and release models could be improved by consistently including moult stage as a variable.

We present a generalized description of the flow of an unspecified metal pollution in a moulting aquatic macroinvertebrate, such as a crustacean. This conceptual description offers an illustrative, non-specific picture of the potential impact of moulting on measured accumulations, equivalent to a repeating, discontinuous depletion of total accumulated metal concentrations. We demonstrate the applicability of this conceptual model by comparing to previously published pre- and post-moult measurements of overall body concentrations of crustaceans, and discuss the relevance of this conceptual model to the study of accumulation in biomonitor species. We also present a possible technique for reducing this variability, and discuss the potential application of this technique both in the field and the laboratory.

CONCEPTUALIZING THE IMPACT OF MOULTING

Existing models

Common types of models used to describe the accumulation of metals in aquatic invertebrates include bioconcentration, bioaccumulation, and accumulation factors (BCF/BAF/ACF), the biotic ligand model (BLM), the free ion activity model (FIAM), and biodynamic models (Wang & Tan, 2019).

BCF, BAF, and ACF factors provide an intuitive and relatively uncomplicated way of estimating accumulation rates, but rely on the assumption that equilibrium will be reached across the organism-environment interface (McGeer *et al.,* 2003; van den Brink *et al.,* 2019). Moulting consists of dynamic changes in the organism, which violates this key assumption, reducing the contribution of moulting to a static correction term rather than a time-varying process.

BLM, as well as the related FIAM and extensions thereof, are commonly applied to studies of the total accumulated concentration ionic metals in aquatic macroinvertebrates (Brown & Markich, 2000; Di Toro *et al.,* 2001; van den Brink *et al.,* 2019). Both models, however, focus on the interface between the environment and the proposed receptor site, and ignore the internal mechanisms of translocation, transformation, and excretion (Vijver *et al.,* 2004). The contribution of moulting is closely linked with the relative sequestration of metal pollutants in exoskeleton and soft tissue compartments, which relies on these internal mechanisms.

Biodynamic models, commonly referred to as physiologically-based pharmacokinetic (PBPK), are better suited to modelling the dynamic potential contribution of moulting to the accumulated concentrations of contaminant (Ardestani *et al.,* 2014; van den Brink *et al.,* 2019). This approach models the processes of uptake, accumulation, translocation, transformation, and excretion across time, and does not rely on any steady-state assumptions. toxicokinetic-toxicodynamic (TKTD) models, such as general unified threshold model of survival (GUTS) (Jager *et al.,* 2011; EFSA Panel on PPR *et al.,* 2018), are an example of a biodynamic approach applied to both contaminant accumulation and resultant biological effects. One of the most comprehensive static metal-accumulation biodynamic models in the literature arguably shows a relatively good correlation with observed results across a large number of studies (Luoma & Rainbow, 2005), and GUTS is considered sufficiently developed for use in risk assessment

142 applications (EFSA Panel on PPR *et al.*, 2018). These models, however, have not yet been
143 extended to include the contribution of moulting, a correction that would have to be separately
144 determined for each organism-analyte pair.
145
146 *Choice of approach*
147 We take the approach of describing moulting using a non-specific model that is designed to
148 capture the essence of the problem, while remaining broadly applicable to any moulting aquatic
149 invertebrates such as crustaceans, whose exoskeleton may act as a significant sink for
150 contaminants, and any transition metal, metalloid, or heavy metal species. Such approach should
151 be contrasted with the common approach deriving a quantitative, predictive model described
152 above. The aim of our model is instead to illustrate certain contributions of the moulting process
153 to measured concentrations that are common to all moulting aquatic invertebrates and metal
154 analytes, without offering a prediction for the significance of these contributions in any one
155 scenario.
156 We limit the applicability of the presented model to the transition, metalloid and heavy
157 metals, as it has been observed that the accumulation of the alkali or alkaline earth metals in the
158 exoskeleton of an aquatic crustacean may differ from that of the aforementioned elements. The
159 accumulation of calcium, in particular, has been extensively investigated throughout the various
160 moult stages, and has been found to undergo a series of storage and resorption processes. This is
161 said to be linked to the use of calcium in the release of the exoskeleton and hardening of the
162 newly developing cuticle (Greenaway, 1985). The chitinous nature of the exoskeleton, and, more
163 specifically, the nitrogen groups therein, may play a role in the alternative behaviour of the
164 transition metals, metalloids, and heavy metals, as it has been noted that chitinous materials

show a poorer affinity towards the alkali and alkaline earth metals (Rae & Gibb, 2003). For this reason, the assumptions made in the following section apply only to the accumulation of transition metals, heavy metals, and the metalloids.

Assumptions

For the purpose of creating a concise and simplified conceptual model, we must introduce a number of assumptions. These assumptions are chosen such that they adequately isolate the impact of moulting on trace metals concentrations, while removing internal processes that are not mediators of the moulting process.

1) In order to reduce the process to a flow network, we make the assumption that all metal pathways are uni-directional from intake to depuration. This does not mean that there are no bi-directional pathways, but rather that bi-directional flows can be replaced by a long-term uni-directional approximation.

2) While the process of moulting may be complex and irregular, we assume that each moulting event happens similarly and that the properties of each moulted exoskeleton are largely identical, in that each sequential exoskeleton is capable of accumulating metal contaminants at a fixed rate, after consideration of the growth factor. This is a simplifying assumption, and the impact of moulting is qualitatively similar under non-uniform moulting behaviour.

3) The frequency of moulting is taken to be constant, for the purposes of illustration. Again, non-constant frequency of moulting would produce qualitatively similar results.

4) Contaminant intake occurs solely through the processes of respiration, ingestion, and adsorption. The inclusion of these three pathways is intended to make the model as general as possible, and the results still hold if uptake through either ingestion or respiration does not occur,

and/or if uptake through adsorption does not occur. Adsorption is defined as the uptake of metal contaminants directly from the overlying and interstitial waters in direct contact with the surface of the exoskeleton, and results in uptake of the contaminant directly into the pre-moult exoskeleton; absorption through the exoskeleton and into the body is not directly considered for the reasons explained in Assumption 1.

5) The only process of depuration included in the model is moulting. Gut contents are not taken into account, and, therefore, excretion from the alimentary tract does not reduce accumulated concentrations in the model; metal pollutants are taken to enter the system when they are assimilated from the alimentary tract into the biological tissues.

6) This model only considers the movement of trace metals and assumes that no internal processes of biotransformation are taking place. This is true regardless when considering the elemental concentrations, but a more complex model would be required to account for change in speciation or complexation of metals due to biological processes.

7) In this conceptual system, we make the assumption that the rate of translocation between the body and pre-moult exoskeleton is driven towards equilibrium by the presence of open binding sites in the destination and high concentrations at the source. The flow of translocation can therefore be approximated as proportional to the source concentrations. Other models of translocation could be considered and would result in qualitatively similar results.

8) We assume that the described processes are not influenced by any biological damage that may occur, and we do not account for the possibility of mortality as part of the model.

The conceptual model

Based on the above assumptions, we present a simplified three-input, two-compartment model of metal accumulation in a hypothetical moulting aquatic invertebrate (Fig. 1). The corresponding rate diagram is shown in Figure 2. The model compartmentalizes metal concentrations accumulated within (and on) the pre-moult exoskeleton (Compartment *E* in Figure 2), which is defined as the part of the body that is removed entirely during the moulting process, and concentrations accumulated in the remainder of the body (Compartment *B* in Figure 2). For the purposes of simplifying the model, the body is inclusive of all non-moulting parts (gills, legs, hepatopancreas, and other organs), but not the gut contents as explained in Assumption 5.

<Figs. 1 & 2>

Contaminants may enter Compartment *B* through ingestion or standard respiration, where *i* denotes the concentration of contaminant present in the ingestate, *r* the concentration of contaminant in the water overlying the gill regions, and k_i and k_r the rate constants of the respective processes. Contaminants may enter Compartment *E* through surface adsorption directly from overlying and interstitial waters in contact with the exoskeleton, where *a* denotes the concentration of contaminant present in these waters, and k_a the corresponding rate constant. Contaminants may also flow from Compartment *B* to Compartment *E* through the process of internal translocation or sequestration, where *B* is the concentration of contaminant in Compartment *B*, and k_t the rate constant of translocation. The features of this flow are summarized in Assumption 7.

Moulting, or ecdysis, refers to the regular removal of the outer exoskeleton. As the exoskeleton is shed, a new exoskeleton develops beneath it (see Drach, 1967). The loss of the moulted exoskeleton cannot be modelled as a continuous flow of contaminants as it occurs suddenly and periodically, and, therefore, it is not included in the process diagram in Figure 2.

Instead, the diagram describes the inter-moult movement of contaminants, and moulting is implemented externally as a periodic discontinuous removal of the contaminants within Compartment *E*. The moult period, or the rate at which an organism moults, will vary greatly with species and other factors. It should be noted that occasional consumption of the organism's own moulted exoskeleton has been observed in some macroinvertebrate species (Elangovan *et al.*, 1999), although there is consensus that metals bound within a chitinous exoskeleton are less bioavailable than other forms of analyte (Khan *et al.*, 2010). The possibility of such an occurrence is not considered in this model but would result in qualitatively similar results.

Impact of moulting

The conceptual two-compartment model of Figure 1 can be converted into a causal diagram describing the relationship between the variable of interest, namely the environmental concentration of bioavailable metal, and the measured metal concentration. This diagram is shown in Figure 3. The effect of environmental concentration on measured concentration occurs through the mediation variables of body and exoskeleton concentration. The hypothesis that the measured whole-body concentration is an accurate estimator for the bioavailable environmental concentration, given an acceptable measurement error, is, therefore, weakened by the direct effect of moult stage on exoskeleton concentration.

<Fig. 3>

It can be directly observed from Figure 3 that the effect of the moult event on the overall accumulated metal concentration depends greatly on the ratio of exoskeleton metal concentration to body metal concentration. If a simplifying assumption is made that the moult stage of different

organisms is uncorrelated, then sampling more organisms should reduce, but not eliminate, the influence of moulting.

DERIVATION OF KEY EQUATIONS

Differential rate equations

The key aspects of the model are described by the following pair of differential rate equations, which describe the change in inter-moult concentrations of metal contaminant in each compartment.

$$\frac{\mathrm{d}[B]}{\mathrm{d}t} = k_i[i] + k_r[r] - k_t[B] \quad (1)$$

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = k_t[B] + k_a[a] \quad (2)$$

Growth factor

The growth aspect is one of significant variability, as growth may indicate linear growth, lateral growth, or increasing thickness of the exoskeleton. Growth rate will vary considerably with species, as well as with the life stage of the organism. In order to account for the growth uncertainty, we use a growth factor, *G*. Equations 1 and 2 can, hence, be extended to account for growth by dividing each k_x term by *G*.

Closed-form expressions

Equations 1 and 2 can be solved for instantaneous compartment concentrations, assuming all concentrations are 0 at time $t = 0$ and ignoring the effects of moulting. This produces the following closed-form expressions:

$$[B](t) = (U/k_t)(1 - e^{-t/\tau}) \quad (3)$$

$$[E](t) = (U + k_a[a])t - U\tau(1 - e^{-t/\tau}) \quad (4)$$

where the uptake rate is given by $U = k_i\,[i] + k_r\,[r]$, and the process time constant is $\tau = G/k_t$.

Note that the concentrations in Compartment *B* can be modelled as a first-order underdamped system, where the concentration approaches an equilibrium value of U/k_t, while the concentration in Compartment *E* increases indefinitely as the loss due to moulting is not yet accounted for.

Steady-state equations

Steady-state is reached when $t \gg \tau$ in all the above equations. This results in expressions for the final, steady-state accumulated concentration in Compartment *B* in an environmental equilibrium.

$$[B] = U/k_t \quad (5)$$

As moulting happens periodically, the steady-state equivalent in the case of the Compartment *E* has the appearance of a sawtooth pattern, rather than a fixed value. The period of the concentration in Compartment *E* is equal to the moulting period, T_M, while the peak concentration is given by:

$$[E]_{\mathrm{MAX}} = (U + k_a[a])T_M \quad (6)$$

The variance of the corresponding error due to moulting is therefore given by:

$$\sigma^2 = \frac{([E]_{\mathrm{MAX}})^2}{12} \quad (7)$$

More explicit derivation of the above equations is presented in Supplementary material File S11.

SIMULATION

Objectives

The conceptual model is designed to offer insights into the contribution of moulting to whole-body concentrations in the general case. The following simulation is intended to provide an example of how the model can describe the impact of moulting in an existing experimental study. Its specificity to a particular organism and metal pollutant should not be taken to be a statement about the limitations of the conceptual model, but rather an indication of how the generalized model can be applied to a specific case. The implementation of the model employed in the following sections is described in full in Supplementary material File S12.

Simulation parameters

The simulation parameters shown in Table 1 were derived from studies of the uptake of vanadium by the caridean shrimp *Lysmata seticaudata* (Risso, 1816) (Miramand *et al.*, 1981). The measurements extracted from Miramand *et al.* (1981) is available in Supplementary material Table S1. Further details of how these parameters were derived are presented in Supplementary material File S13 and compared with the cited measurements in Supplementary material Fig. S8.

<Table 1>

Simulation results

Figure 4 shows the bioaccumulated concentrations of metal contaminant in Compartment *B*, when the environmental conditions are in equilibrium. The black dashed lines indicate the steady-state values.

<*Fig. 4*>

As expected of a first-order system, the concentration in Compartment *B* reaches steady-state at a speed dictated by the rate of internal translocation of the contaminant. Once the internal concentration reaches steady-state, there is no significant change in contaminant concentration without a corresponding change in the environmental conditions.

Figure 5 shows the bioaccumulated concentration of the contaminant in Compartment *E*. Moulting forces the pre-moult exoskeleton concentration to 0 every T_M days. This produces a periodic pattern with increasing amplitude, approaching a sawtooth pattern as Compartment *B* reaches steady-state.

<*Fig. 5*>

The total concentration of contaminant in the organism as a whole is shown in Figure 6. The lower black dashed line shows the steady-state concentration for Compartment *B*, whereas the upper black dashed line includes the peak concentration for Compartment *E*. Despite the concentration in Compartment *B* reaching steady-state, the influence of moulting is still significant. This results in a time variation in the overall concentration.

<*Fig. 6*>

DISCUSSION

Contribution of moulting

Studies measuring the bioaccumulation of trace metals in freshwater macroinvertebrates typically rely on the assumption that the accumulated concentrations of metals are relatively time-invariant. Many models of pollutant uptake likewise rely on the steady-state assumption. In both cases, individual measurements of total accumulated pollutant concentrations provide an accurate quantification of the time-averaged accumulation flux.

The contribution of the conceptual model presented herein is to explain how periodic processes, exemplified by moulting, can produce fluctuations in the total accumulated whole-body concentrations. The *L. seticaudata* example demonstrates the significant effect this can have on the measured whole-body concentration. Seeing as discontinuous loss occurs during each moult, steady-state is not reached; the internal concentrations settle into a periodic oscillation, with the total concentration varying between minimum and maximum values. The error introduced by the continuous approximation is described in Supplementary material File S14, and illustrated by Supplementary material Figs. S9, S10. From an experimental perspective, this introduces a source of variability in the measurements, as the measured value depends not only on the mean total concentration, but also on the moult stage at the time at which the measurement is taken.

Validity of simulated example

Example parameters, listed in Table 1, were used for the purposes of demonstrating the effects of moulting on the measured whole-body concentration. This raises the question of whether the chosen parameters produce results that fairly represent realistic pollutant accumulations. This can only be answered through comparison with measured concentrations.

364 The model predicts that approximately 74% of the accumulated metal contaminant concentration
365 is lost during the process of moulting. Table 2 presents published measurements of analyte
366 concentrations accumulated within the exoskeleton of various species, expressed as a percentage
367 of whole-body accumulated concentrations. In cases where data extraction and/or post-
368 processing was required to obtain the values given in Table 2, further details are given in
369 Supplementary material Tables S4–S7. It must be emphasized, when interpreting these figures,
370 that most of the cited studies do not account for the effects of moulting on overall concentrations
371 we describe. Therefore, by assuming steady-state whole-body concentrations it would be
372 expected that the measured values presented herein represent approximately half of the
373 exoskeleton concentration at the time of moulting. Under these same assumptions, the
374 exoskeleton would contribute approximately 37% of the whole-body concentration using the
375 model parameters presented herein.

376 As is to be expected and considering the wide range of biological species, analytes, and
377 environmental or experimental conditions, there is a broad variation in reported exoskeleton
378 concentrations. Despite this variation, it is clear that such concentrations are a significant fraction
379 of the whole-body accumulated concentration. Our model would, therefore, be correct in
380 attributing a significant role to the contribution of moulting to whole-body pollutant
381 concentrations.

382 *<Table 2>*

383 If the results of the model are valid, the question then arises as to whether the
384 mechanisms described in the model are also valid. Hall (1982) presented measurements of the
385 accumulated concentrations of nickel in the cladoceran *Daphnia magna* Straus, 1820, presented
386 here in Supplementary material Tables S2, S3. Figure 7 shows the measured soft-tissue and

exoskeleton concentrations in individuals that have not moulted. It shows that the soft-tissue concentrations rapidly reach steady-state, while the exoskeleton concentrations continue to increase in the absence of moulting. A corresponding fit of the model is shown, with a value of $P < 0.001$ for both datasets. *In-vivo* measurements of whole-body nickel concentrations of one individual over time are shown in Figure 8. A moulting event occurred between t = 20 h and t = 49 h, depleting the whole-body concentration. Our model correctly describes the effects due to this moulting behaviour. These results indicate that our description of the processes that result in depuration via moulting is likely valid. Further details of the derivation of these parameters is given in Supplementary material File S15.

<Figs. 7 & 8>

Reducing variability due to moulting

Figure 9 shows how accumulated concentrations can fluctuate through time. For most of the moult period it is unclear at what point in the period the organism lies. In the context of crustaceans, however, it is usually relatively easy to identify if the organism is immediately at a pre-moult or post-moult stage (Drach, 1967; Buchholz, 1982). The pre-moult stage is often, depending on the species, associated with visual changes to the exoskeleton, such as changing colour or texture (Drach, 1967). The post-moult stage is, at the very least, signalled by the appearance of a shed cuticle. Both these stages correspond to the maximum and minimum exoskeleton concentrations, respectively. In the context of bioaccumulation studies, we therefore propose that sampling could be undertaken synchronously with moulting (moult-synchronous sampling) to ensure the robustness of the measurement by reducing the variability due to

409 exoskeleton concentration fluctuations. This would take the form of ensuring only specimens
410 which are immediately pre-moult or post-moult are sampled.

411 <*Fig. 9*>

412 In the context of *ex-situ* studies, implementation of moult-synchronous sampling quite
413 simply takes the form of delaying sampling until the desired moult stage has been reached for
414 each organism. Implementation for *in-situ* studies, however, is less straightforward due to the
415 requirement that samples be taken when the site is visited. In a case such as this, we propose
416 acquiring the organisms as normal, but holding them in a suitable tank until the desired moult
417 stage has been reached. This approach assumes that minimal depuration through other means
418 occurs between acquisition and moulting.

419 An argument could be put forward that the variability due to moulting could be reduced
420 by sampling multiple specimens. This argument relies on the assumption that the moult stage of
421 each specimen is uncorrelated; however, moulting can be induced or accelerated by
422 environmental stressors (Fowler *et al.,* 1971; Nugegoda & Rainbow, 1987), therefore, it could
423 happen that specimens in similar conditions can moult together. For this reason, performing
424 measurements on multiple specimens held in the same conditions may not be sufficient to
425 overcome the effects of moulting on accumulated concentrations.

426 Even ignoring the possibility of correlated moult stage within the population under study,
427 moult-synchronous sampling can offer a more efficient approach to the determination of mean
428 total bioaccumulated concentrations. Both increased sampling size and moult-synchronous
429 sampling aim to reduce the measurement error in the overall measured accumulated
430 concentration due to moulting. Equation 7 quantifies the variance of the measurement error due
431 to moulting. In the example of Figure 6, this corresponds to a mean error variance of $\sigma^2 = 14$

ppb. If measurements were only made within the first post-moult day, this would reduce the mean error variance of a single measurement to $\sigma^2 = 0.14$ ppb. In lay terms, the resulting increase in statistical accuracy corresponds to that which would be obtained by increasing the number of specimens by a factor of 100. Figure 9 shows the reduction of measurement error variance due to moulting from restricting the sampling window. Moult-synchronous sampling is, therefore, a more efficient means of reducing the measurement error due to pollutant loss during moulting than simply increasing the sampling size.

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SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Accumulated vanadium concentration values derived from Miramand *et al.* (1981).

S2 Table. Accumulated nickel concentration values derived from Figure 6 of Hall (1982).

S3 Table. Accumulated nickel concentration values derived from Figure 2a of Hall (1982).

S4 Table. Accumulated concentration values derived from Bergey & Weis (2007).

S5 Table. Accumulated concentration values derived from Hennig (1984).

S6 Table. Accumulated concentration values derived from Keteles & Fleeger (2001).

S7 Table. Accumulated vanadium concentration values derived from Reinecke *et al.* (2003).

S8 Figure. Accumulation data from Miramand *et al.* (1981) (CF actual), and fit to Equation 3 (CF fit), with T_M = 21 days and G = 1.

S9 Figure. Simulated exoskeleton compartment pollutant concentration, with continuous moulting approximation (given by Equation S14) overlaid in dashed black.

S10 Figure. Error in whole-body pollutant concentration when modelling moulting as a continuous process.

S11 File. Expanded derivations of the model equations.

S12 File. Implementation of the model code.

S13 File. Derivation of model parameters from data presented in Miramand *et al.* (1981).

S14 File. Description of the continuous approximation assumption and resulting error.

S15 File. Derivation of model parameters from data presented in Hall (1982).

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FIGURE CAPTIONS

Figure 1. Schematic representation of a general overview of the major pathways of trace metal pollutant uptake (via ingestion, respiration and adsorption), translocation, and loss (via moulting) in a moulting aquatic organism. The dashed arrow represents the elimination of accumulated pollutants at the time of moulting, which is modelled as a repeating instantaneous event, while the solid arrows represent the continuous pollutant flux.

Figure 2. Rate diagram of pollutant flux into and out of the body (*B*) and moulting exoskeleton (*E*) compartments. Processes illustrated are respiration (*r*), ingestion (*i*), adsorption (*a*), and internal translocation (*t*); *r*, *i*, and *a* represent the concentrations from which respiration, ingestion and adsorption, respectively, occur, *B* the body compartment concentration. The k_x parameters represent the respective rate constants.

Figure 3. Causal diagram showing the connection between bioavailable environmental metal concentrations and measured whole-body metal concentrations, showing the influence of moult stage on the exoskeleton concentration mediator.

Figure 4. Simulation of theoretical concentration of a metal pollutant in Compartment *B* (the body of the organism, excluding the moulting exoskeleton) versus time, from application of the described parameters. The dashed black line represents the equilibrium value given by Equation 5 (see text). The lighter lines represent the effects of different values of k_t for the same equilibrium value.

Figure 5. Simulation of concentration of a metal pollutant in Compartment *E* (the moulting exoskeleton of the organism) versus time, from application of the described parameters. Unlike with the concentration in the body compartment, *B* (see Fig. 2), the concentration does not reach an equilibrium state, but oscillates between 0 (complete absence, due to moulting) and a maximum value. The equilibrium maximum value is given by Equation 6 (see text), and is denoted here by the dashed black line.

Figure 6. Simulation of overall concentration of a metal pollutant in the organism *versus* time. The overall concentration is a mass-weighted combination of the concentrations in the body and moulting exoskeleton compartments, *B* and *E*. This simulation represents the evolution of the actual measured whole-body concentration over time, where the variation in the concentration beyond day 200 is entirely due to contaminant loss through moulting. The equilibrium minimum and maximum are given by the dashed black lines, and are derived from Equations 5 and 6 (see text) after accounting for body mass.

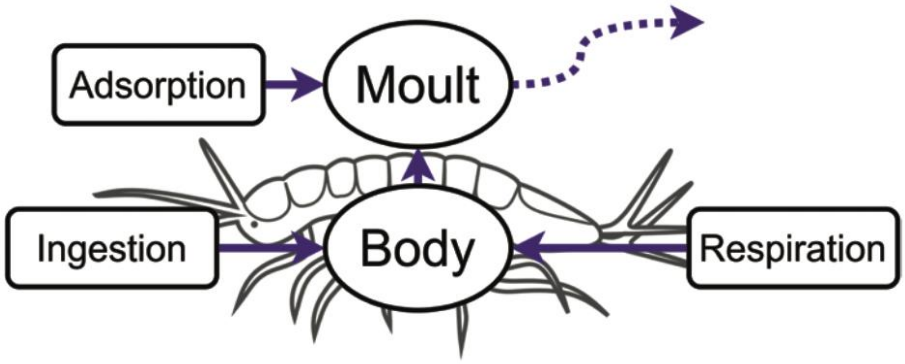
Figure 7. Accumulated concentrations of nickel in the soft-tissue (*B*) and exoskeleton (*E*) of multiple *Daphnia magna* individuals (from Hall, 1982). The corresponding fit of the model qualitatively matches the behaviour seen, where *B* saturates, but *E* continues to accumulate indefinitely in the absence of moulting. Hall (1982) also observed indefinite accumulation in the

filtering appendages, which contain parts of exoskeleton and soft tissue, so have not been included in this figure.

Figure 8. Accumulated concentrations, across time, of nickel in an individual specimen of *Daphnia magna* (from Hall, 1982). A moulting event occurred between t = 20h and t = 49h. A fit of the model, accounting for a moulting event just before t = 49 h, accurately describes the observed behaviour, despite the relative simplicity of the model.

Figure 9. Proportional decrease in moult-induced measurement error with increasing accuracy of moult-synchronous sampling, where a "100%" sampling window is equivalent to ignoring moult stage when sampling. Using a sampling window of 10% of the moult period, for example, would reduce the variance by a factor of 100, for the same sample size.

669 **Figure 1.**



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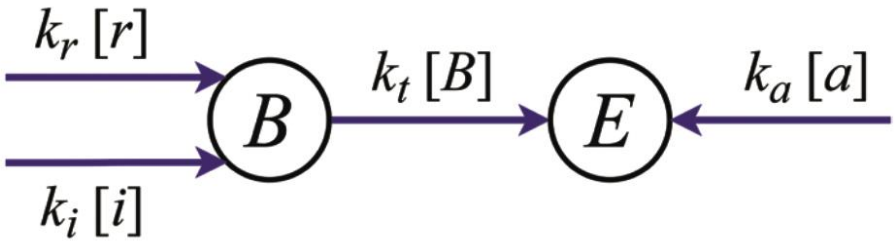

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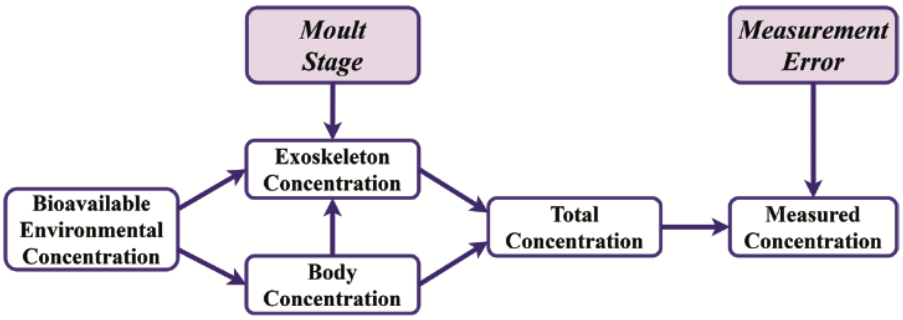

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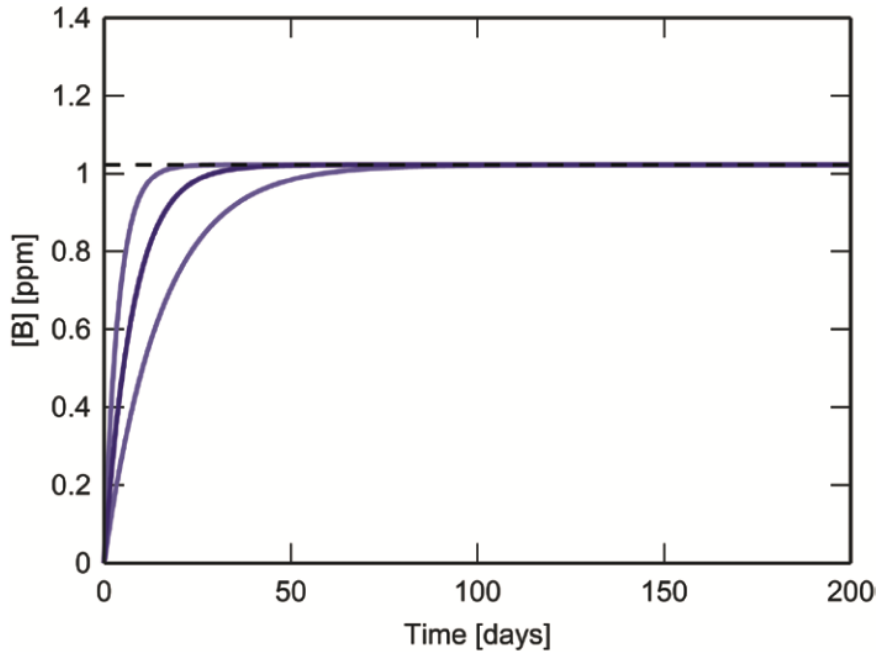

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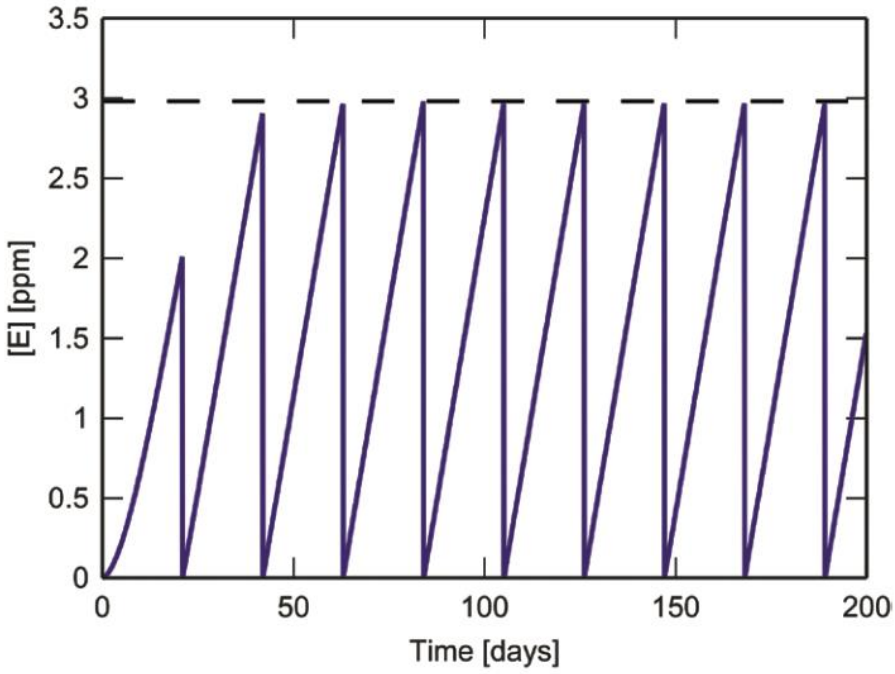

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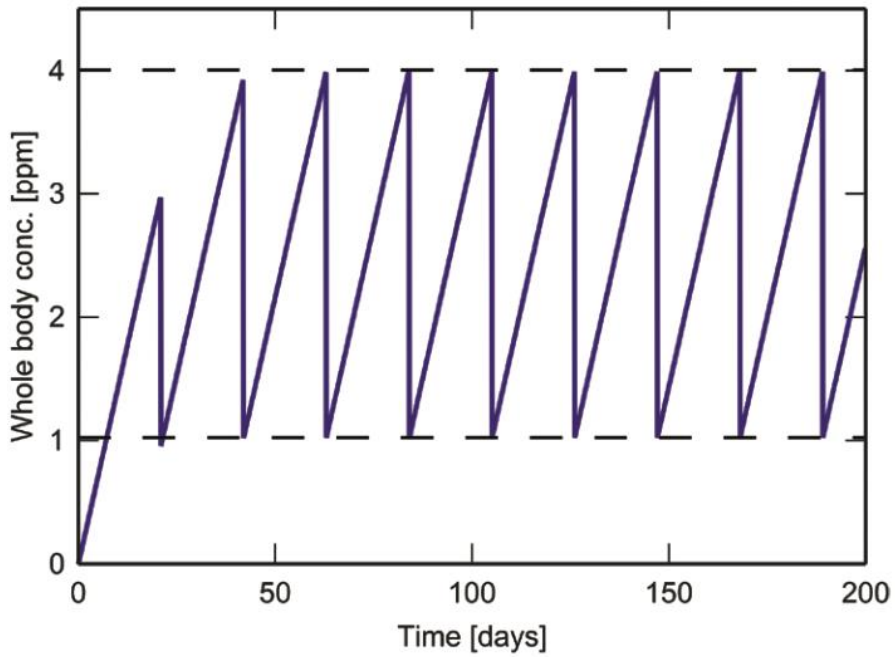

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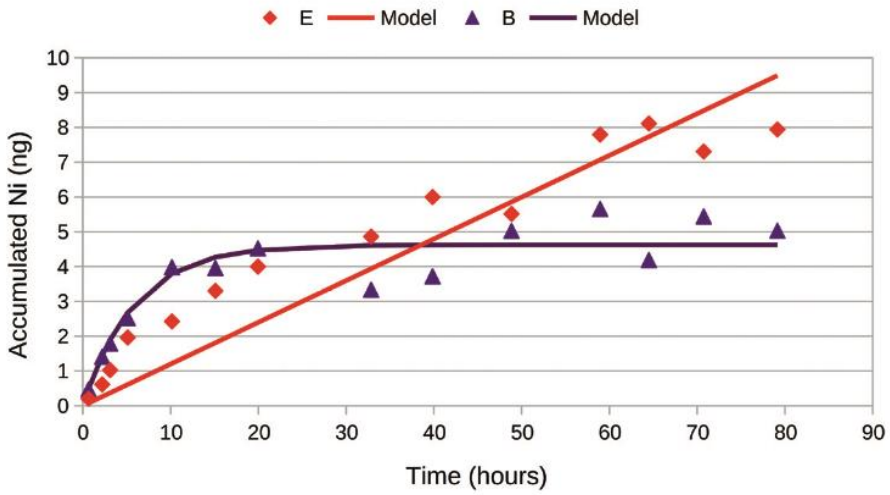

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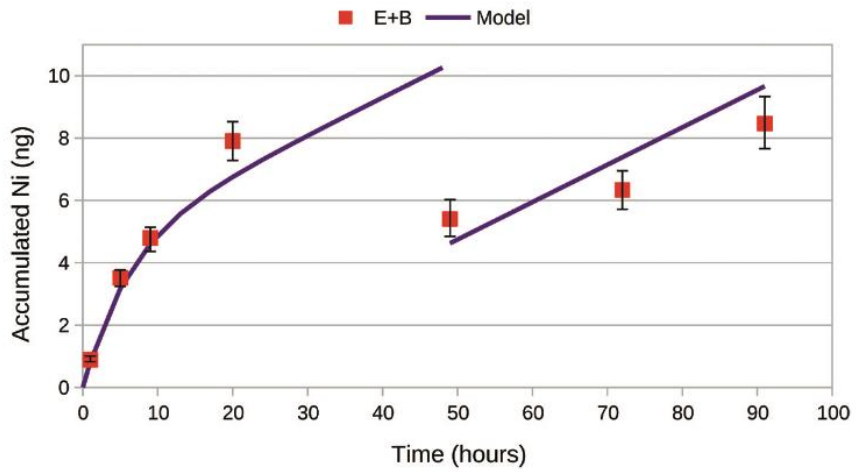

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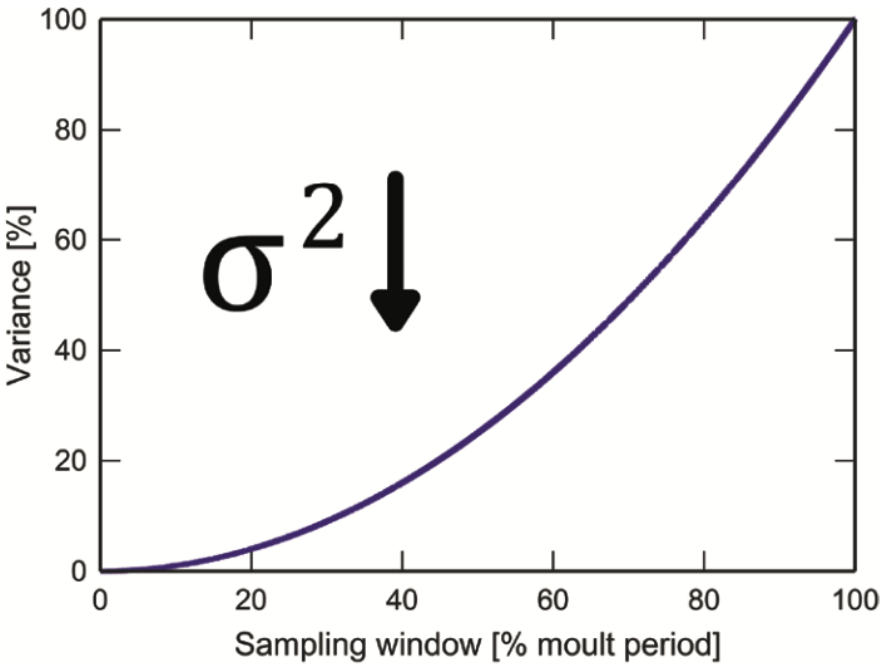

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